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Survival of the Fittest: How Bacterial Pathogens Utilize Bile To Enhance Infection

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SUMMARY

Bacterial pathogens have coevolved with humans in order to efficiently infect, replicate within, and be transmitted to new hosts to ensure survival and a continual infection cycle. For enteric pathogens, the ability to adapt to numerous host factors under the harsh conditions of the gastrointestinal tract is critical for establishing infection. One such host factor readily encountered by enteric bacteria is bile, an innately antimicrobial detergent-like compound essential for digestion and nutrient absorption. Not only have enteric pathogens evolved to resist the bactericidal conditions of bile, but these bacteria also utilize bile as a signal to enhance virulence regulation for efficient infection. This review provides a comprehensive and up-to-date analysis of bile-related research with enteric pathogens. From common responses to the unique expression of specific virulence factors, each pathogen has overcome significant challenges to establish infection in the gastrointestinal tract. Utilization of bile as a signal to modulate virulence factor expression has led to important insights for our understanding of virulence mechanisms for many pathogens. Further research on enteric pathogens exposed to this *in vivo* signal will benefit therapeutic and vaccine development and ultimately enhance our success at combating such elite pathogens.

INTRODUCTION

Enteric pathogenic bacteria represent a unique set of pathogens, as these bacteria must overcome numerous challenges in order to successfully establish infection in the small intestine or colon (Fig. 1). These challenges include pathogen-specific immune responses (1) and several bactericidal conditions of the gastrointestinal tract, including the low pH of the stomach (2), the presence of bile in the small intestine (3, 4), low iron availability (5, 6), and
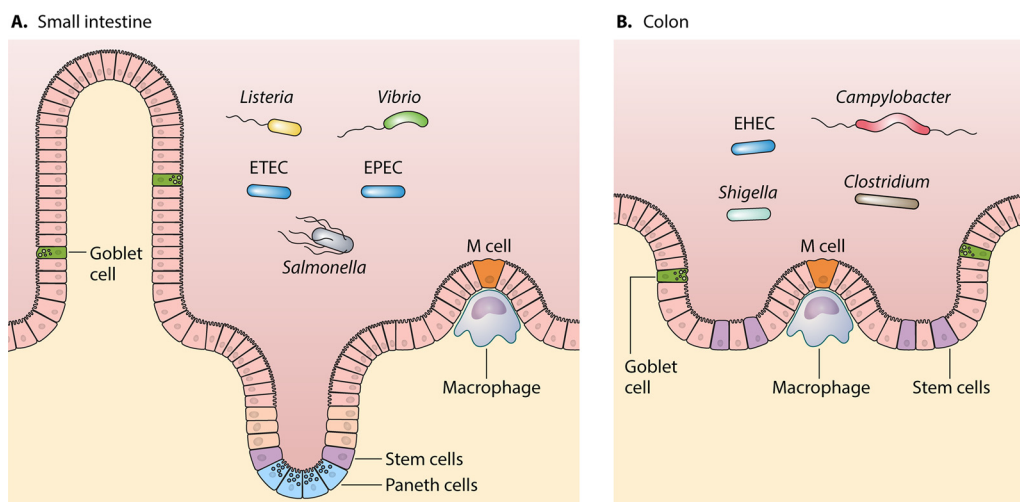
FIG 1 Infection sites of enteric pathogens in the gastrointestinal tract. Enteric bacterial pathogens have been shown to infect either the small intestine (represented with microvillus protrusions) (A) or the colon (B). In both settings, goblet cells produce mucus, stem cells initiate maturation of the enterocytes, and specialized microfold (M) cells serve as the point of antigen sampling that can be exploited by invasive pathogens to access epithelial cells. Paneth cells in the small intestine are important for initiating innate mucosal defenses. Infection mechanisms include adherence to or invasion of the epithelial cells lining the gastrointestinal tract, with *Listeria*, *Vibrio*, *Salmonella*, and some pathogenic *Escherichia coli* strains (EPEC and ETEC) infecting the small intestine and *Campylobacter*, EHEC, *Shigella*, and *Clostridium* infecting the colon.

an established commensal microbiome consisting of billions of bacteria from hundreds of different species (7). The human gastrointestinal tract is a challenging environment that prevents many bacteria from surviving under these harsh conditions; however, enteric bacterial pathogens have evolved not only to survive these severe exposures but also to successfully colonize and establish infection in the host.

Given the length of the small intestine, bile is one of the more long-term exposures encountered by bacteria, both pathogenic and commensal, in the early part of the gastrointestinal tract. For the host, bile is essential for digestion and nutrient absorption and is composed of proteins (globulins and albumins), lipids (phospholipids, cholesterol, and fatty acids), carbohydrates, vitamins, mineral salts, and other trace elements (8). In the liver, the primary bile acids cholic acid and chenodeoxycholic acid are synthesized by hepatocytes and are further metabolized in the liver by conjugation to glycine or taurine through *N*-acyl amidation. Conjugated bile acids have reduced bile acid toxicity and increased solubility, are almost fully ionized at physiological pH, and are termed bile salts. Bile salts are maintained at high concentrations in the duodenum, jejunum, and proximal ileum to solubilize, digest, and absorb lipids and lipid-soluble vitamins (8, 9). The facultative and anaerobic commensal bacteria in the small intestine metabolize bile salts through deconjugation and hydroxy group oxidation, resulting in the production of secondary bile salt compounds. In the distal ileum, bile salts are absorbed into the bloodstream, complexed to plasma proteins, and returned to the liver (9). The majority of bile (95%) is recycled back into the small intestine and does not enter the colon (10). Concentrations of bile salts range from 0.2% to 2% (wt/vol), depending on the time of day, diet, and individual (11). As reviewed by Ridlon et al., bacterial colonization in the small intestine increases from ~10^3 bacteria/ml in the duodenum to 10^4 bacteria/ml in the jejunum and 10^6 to 10^8 bacteria/ml in the ileum. *Lactobacillus* and *Streptococcus* are the predominant genera in the duodenum and jejunum, while

Enterococcus, *Bacteroides*, *Clostridium*, and other commensals, in addition to *Lactobacillus*, are able to colonize the ileum (9). Human and rodent studies have demonstrated that the bactericidal properties of bile components likely prevent overgrowth of the commensal bacteria in the small intestine. The absence of this mechanism is problematic for individuals who have reduced bile output, such as patients with hereditary defects like intrahepatic cholestasis (12–15).

Depending on the organism and the site of infection, pathogenic bacteria have developed resistance mechanisms to counteract the bactericidal conditions of bile. In fact, a number of enteric pathogens use bile as a signal to regulate virulence gene expression to either colonize or maintain infection in the human gastrointestinal tract (Tables 1 and 2). For pathogens that colonize the small intestine, resistance to bile is critical to ensure long-term survival in the host. Here we review the bile-associated regulation of virulence mechanisms in enteric bacterial pathogens and provide an analysis of the bile-related research performed to date.

ESCHERICHIA COLI

Escherichia coli is a Gram-negative, facultative, anaerobic bacterium that naturally resides in the gastrointestinal tract of mammals and composes 0.1% of the microbiota found in the cecum and colon of humans (16). *E. coli* strains can include both nonpathogenic commensals or pathovars associated with gastrointestinal, urogenital, and extraintestinal infections (17). As *E. coli* bacteria traverse the gastrointestinal tract, they encounter signals provided by the host that trigger the expression of numerous colonization mechanisms, a process known as interkingdom signaling (18). These signals include factors that facilitate gastrointestinal adherence of the bacteria to the small intestinal or colonic epithelial cells (17, 19). Studies investigating bile responses in *E. coli* include both commensals and diarrheagenic pathovars, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enterohemorrhagic *E. coli* (EHEC) (20–28).

TABLE 1 Summary of bile resistance mechanisms in enteric pathogens

Pathogen	Function of outer membrane proteins (reference[s])	Mechanism of induction of stress response genes (reference[s])	Efflux pump(s) (reference[s])	Additional resistance mechanism(s) (reference[s])
Escherichia coli	Repression of *ompF* (23)	*sulA* to correct DNA damage (35)	AcrAB (23, 38)	MqsR/MqsA toxin/antitoxin system (34); *basRS* two-component system (23)
Shigella	To be determined			
Vibrio	Regulated expression of *ompU* and *ompT* (76)	RpoS in *V. vulnificus* (102)	AcrAB (77, 78), BreAB (79, 80), VexAB (81, 101), VprAB (82), Vme pumps (99, 100)	Induction of transcriptional regulator LeuO (75) and biofilm formation (84)
Salmonella	Repression of OmpF and OmpC (3); utilization of TolA and TolC (3, 125)	SoxRS (132) and OxyR (133)	AcrAB (119–121); AcrEF, MdtABC, MdsCBA, EmrAB, MdtK, and MacAB (118)	Alterations of LPS, including remodeling of lipid A (3), elongation of O-chains through *fepE* (129), and Vi antigen production (130)
Campylobacter			CmeABC and CmeDEF (154, 155)	Utilization of the two-component regulator CbrR (159)
Clostridium	To be determined; spores are naturally resistant (169)			
Listeria		Induced stress response genes encoding DNA repair proteins, protein folding chaperones, and oxidative stress response proteins (176)	MdrT (177)	BrtA transcriptional regulator (177); bile salt hydrolase enzyme (178); biofilm formation genes induced (35)

Commensal *E. coli* and Common Bile Resistance Mechanisms Shared with Pathovars

Since bile is a major host-produced component encountered by *E. coli* in the small intestine (29), resistance to bile acids in both commensal and pathogenic *E. coli* strains has long been known to be an important property for overcoming the environmental stress of the small intestine, where bile is most abundant (30, 31). A study comparing 72 *E. coli* reference collection (ECOR) strains and 10 EHEC O157:H7 strains demonstrated that commensal and pathogenic strains had similar growth capabilities in sodium dodecyl sulfate (SDS), a detergent often used to mimic bile salts exposure (32). Additionally, a study in mice demonstrated that the presence of bile salts in the gastrointestinal tract is a selective pressure for *E. coli* that confers a fitness advantage to strains that can survive and colonize in the presence of bile (33). In order to counteract the detergent-like properties of bile, both commensal and pathogenic *E. coli* strains activate stress responses, efflux pumps, and toxin/antitoxin systems to remove bile compounds (21, 30, 34). Stress response genes induced by bile salts, such as *sulA*, are used for the cessation of cell division in order to correct damage to DNA and the membrane following oxidative stress (21, 35–37), while efflux pump mechanisms, such as AcrAB-TolC, overcome bile acid toxicity and confer resistance to bile (38). A recent study demonstrated that the MqsR/MqsA toxin/antitoxin system degrades *ygiS* mRNA, which encodes a periplasmic protein that promotes the uptake of the bile salt deoxycholate by the bacteria and increases stress on the bacterial membrane (34). *ygiS* deletion mutants demonstrated improved growth and survival and reduced membrane damage in the presence of the bile salt

deoxycholate (34). Both commensal and pathogenic *E. coli* strains utilize these mechanisms to resist bile; however, the pathogens also carry virulence genes that are tightly regulated in response to bile and other environmental signals present in the gastrointestinal tract in order to outcompete commensal organisms.

Pathogenic *E. coli*

Genome sequencing has demonstrated that the majority of pathogenic strains associated with gastrointestinal infection contain virulence genes often carried on mobile genetic elements that are lacking in commensal strains. Pathogenic *E. coli* bacteria that infect and colonize the gastrointestinal tract encode virulence factors, typically fimbriae and enterotoxins, that aid in bacterial colonization and allow the pathogen to outcompete commensals (17, 19, 25, 39). Diarrheagenic *E. coli* strains can be grouped into six well-defined pathovars characterized by the presence of canonical virulence factors that have been demonstrated to be essential for infection. Isolates of the different pathovars preferentially colonize regions of the gastrointestinal tract, including the small intestine and/or large intestine, to cause disease. Bile responses have been characterized for three of these pathovars: ETEC, EPEC, and EHEC (17, 19, 20, 22–24).

Enterotoxigenic *E. coli*. ETEC, a significant cause of diarrheal illness around the world, is characterized by the production of heat-stable (ST) and/or heat-labile (LT) enterotoxins (40). ETEC isolates can encode >25 described colonization factors for colonization of the small intestine (41–43). Studies of bile responses in ETEC have established that bile salts are an important signal for inducing colonization factor expression in some isolates

TABLE 2 Summary of altered virulence gene expression in the presence of bile

Pathogen	Adhesin/flagellum (reference[s])	Invasin/secretion system (reference[s])	Toxin(s) (reference[s])	Additional factor(s) or description (reference[s])
Escherichia coli				
ETEC	Induced colonization factors such as CS19 (45) and CS5 (44)		Altered *estA* and *eltA* enterotoxin expression (24)	
EPEC	Induction of the bundle-forming pilus, increased adherence, and decreased motility (25)			Reduced bile reabsorption by the apical sodium-dependent bile acid transporter in enterocytes (50)
EHEC	Repressed flagellar motor genes (39)		Repressed Shiga toxin expression (23)	Repressed LEE pathogenicity island (39)
Shigella	Induced adherence mediated by OspE1/OspE2 (65)	Induced invasion through IpaD interaction (61)		Induced protein secretion (58, 65), and 51 induced genes in the presence of bile salts (65)
Vibrio				
V. cholerae	Repressed toxin-coregulated pilus and increased motility (88, 89)	Induced expression of T3SS genes (94); induction of VopX, a T3SS effector (95); diminished T6SS activity (97)	Repression of cholera toxin (88, 89), induction of the related TRH (96)	Effects on transcriptional regulators ToxR (90), TcpP (91), H-NS and FlrA (92), and ToxT (93)
V. parahaemolyticus		Induced expression of T3SS2 (105)	Induced expression of TDH (105)	Induced transcriptional regulators VtrA and VtrB (105); 69 genes induced in the presence of bile (104)
V. vulnificus	To be determined			
Salmonella	Downregulation of motility; regulation of fimbrial adherence factors (128, 137, 142)	Repression of T3SS through SPI-1 inhibition (126, 136); SipD interacts with bile (140, 141)		Repression of SirC and InvF (3, 117); gallbladder colonization
Campylobacter	Increased flagellin A expression (166), biofilm formation mediated through adhesion with the flagellar filament (165)	Induced expression of Cia (164)		Increased chemotaxis through induced CheV protein and ATP synthase expression (166)
Clostridium				Enhanced germination of spores with greater abundance of primary bile acids following antimicrobial killing of the microbiota (170, 172)
Listeria				Possible enhanced fecal shedding following growth in the gallbladder (174)

(24, 44–46). Sommerfelt et al. demonstrated that bile salts exposure significantly increased the fimbriated cell percentage (FCP) in numerous wild-type ETEC strains that were lacking the virulence regulator CfaD (46). Other *in vitro* studies demonstrated bile-dependent fimbrial expression of CS19 (coli surface antigen 19) in ETEC strain F595C via electron microscopy (45), while a transcriptional study by Nicklasson et al. further identified mRNA expression of CS5 fimbriae in response to a bile salt component, sodium glycocholate hydrate, in four different ETEC strains (44). With regard to toxin production, preincubation with crude bile has been demonstrated to have direct inhibitory effects on the ability of the heat-labile toxin protein to bind to the GM1 receptor (47). Overall, these results highlight the significance of the up-regulation of colonization mechanisms by ETEC in response to bile before the initiation of toxin and virulence gene expression in the gastrointestinal tract. Interestingly, a more recent study by

Sahl and Rasko used global transcriptomics techniques to further verify bile salts as a trigger for virulence genes by using quantitative reverse transcription-PCR (qRT-PCR) and transcriptome sequencing (RNA-Seq) in a strain-dependent manner (24). Variations in enterotoxin gene expression between two severe ETEC prototype isolates in response to bile were observed. Prototype ETEC strain H10407 had a significantly greater increase in the expression level of the heat-labile enterotoxin gene *eltA* than did strain E24377A, while the heat-stable enterotoxin gene *estA* was induced in E24377A. Moreover, the colonization factors CS1 and CS3 were downregulated in E24377A in the presence of bile. Analysis of growth at different time points hinted that transcriptional responses were potentially intertwined with a quorum-sensing mechanism (24). This study suggests that some ETEC isolates encode diverse transcriptional networks that can regulate virulence genes differently in response to bile. In all, the responses of ETEC

to bile will require further functional studies to describe the complex interactions in detail, and understanding the bile response pathway in ETEC should prove useful considering the propensity of ETEC to infect the small intestine.

Enteropathogenic *E. coli*. EPEC, an *E. coli* pathovar that is associated with infant diarrhea and the production of attaching and effacing lesions on enterocytes, also colonizes the small intestine (17, 19). EPEC has been demonstrated in numerous studies to respond to bile salts by increasing adhesion to epithelial cells (25, 35, 48). de Jesus et al. demonstrated that bile exposure enhanced the adhesion of typical EPEC strain E2348/69 to Hep2 epithelial cells between 1.3- and 2.6-fold. There was also an increase in bundle-forming pilus protein expression as determined via Western blot analysis (25). Other studies using the major bile component deoxycholate observed decreased flagellar protein levels in EPEC strain E2348/69 and unaltered intimin expression (25, 31). The data suggest that colonization factors other than fimbriae were utilized to enhance binding events in typical EPEC strains in response to bile. Conversely, enhanced adhesion was not observed with atypical EPEC strain 1551-2, which lacks the bundle-forming pilus, when grown on HeLa cells (49). A second interesting observation is that EPEC responses to bile have been demonstrated to impact host cell physiology, as described by Annaba et al. in a study showing that EPEC inhibits enterocyte bile absorption in the ileum and leads to increased bile concentrations in the colon. Caco-2 or Hek293 cells infected with EPEC prototype strain E2348/69 exhibited reduced uptake of taurocholic acid by the apical sodium-dependent bile acid transporter (ASBT), a transporter important for bile absorption (50). Bile malabsorption by enterocytes can result in high bile concentrations in the colon, which can result in mucosal damage and may be associated with chronic inflammation in the gastrointestinal tract (51). However, bile malabsorption was ablated with mutations in the EPEC type III secretion system (T3SS) effectors *espNABD* (known inhibitors of macrophage phagocytosis) or in the presence of protein tyrosine phosphatase inhibitors, suggesting that these effectors function as phosphatases to inhibit the ASBT (50, 52). The interaction of EPEC with host enterocytes appears to be quite dynamic in response to bile and may also impact subsequent disease manifestations. The clinical implication of bile malabsorption during and after EPEC infection requires more investigation in order to understand important implications for therapeutic and vaccine development.

Enterohemorrhagic *E. coli*. EHEC is a diarrheagenic pathovar that has an infectious dose as low as 100 cells and causes disease in the large intestine. EHEC appears to employ responses that are adapted to the colon, a region that contains only 5% of the bile concentrations present in the small intestine (17, 53, 54). Unlike EPEC and ETEC, EHEC isolates do not appear to upregulate colonization factors required for adherence in response to bile but instead have a stress response that is similar to those observed for commensal *E. coli* strains (32). A study evaluating bile salt responses of EHEC O157:H7 using microarrays identified increased expression levels of virulence genes involved in the *acrAB* efflux pump and *basRS* two-component signal transduction system but downregulation of Shiga toxin genes and the outer membrane porin gene *ompF*. Additionally, lipid A modification genes of the *arn* operon were upregulated in bile salts, which, together with the *basRS* system, appear to allow EHEC to resist both bile and the antimicrobial peptide polymyxin B during colonization of the gas-

trointestinal tract (23). A recent study by Hamner et al. reported similar results, demonstrating Shiga toxin gene downregulation when interrogating the bile response transcriptome of O157:H7 by using qRT-PCR. This study observed a significant downregulation of genes in the locus of enterocyte effacement (LEE) pathogenicity island, including the *eae* intimin gene (39). However, this finding was not observed by Kus et al., and the lack of concordance of the results was attributed to variations in the EHEC strains and growth time points used. Furthermore, Hamner et al. identified an increased regulation of genes encoding the iron acquisition and flagellar body segments; however, a 2-fold decrease was detected for genes associated with the flagellum filament, stator motor, and chemotaxis mechanisms. Moreover, no significant transcriptional alteration was observed for the adhesin gene *ompA*, which encodes a protein important for Gram-negative membrane integrity and adherence (39, 54). The downregulation of the LEE and flagellar motor genes coupled with the lack of a change in outer membrane protein genes indicate that O157:H7 may have additional, as-yet-unidentified virulence factors that respond to bile during early-stage pathogenesis or in preparation for colonization of the large intestine. Alternatively, the response to bile may indicate a mechanism used by EHEC as an environmental signal to delay the transcription of genes associated with colonization until the bacteria reach the more permissive environment of the colon. Future transcriptomic studies will enable us to understand the full extent of the response of EHEC to bile and the role that the response plays during EHEC infection.

Future Studies of *Escherichia coli*

There are several areas of research that will improve our understanding of bile responses in both pathogenic and commensal *E. coli* strains. First, future studies should seek to understand whether other understudied pathovars, such as diffuse-adhering *E. coli* (DAEC) and enteroinvasive *E. coli* (EIEC), alter the expression of colonization factors following bile exposure. These studies should also identify the potential link between bile exposure and colonic inflammation that may be associated with the newly defined adherent-invasive *E. coli* (AIEC) pathovar, especially since recent work has demonstrated that bile salts induce the expression of long polar fimbriae to facilitate host cell adhesion (55). Additional genomic and transcriptomic comparisons will provide insight into which components of the bacterium are responsible for bile resistance and the regulation of bile-mediated virulence factors. Furthermore, investigations of how diseases are associated with opportunistic or enteric pathogens could provide clues toward understanding the clinical relevance of *E. coli* in Crohn's disease and other inflammatory bowel diseases. Comparative studies of various diarrheagenic pathogens should seek to address colonization efficiency to identify if these differences account for the establishment of the different niches along the gastrointestinal tract exploited by enteric pathogens. Finally, it remains to be seen if the ability of *E. coli* to resist bile is significantly different between commensal and pathogenic strains, as very few commensal isolates have been studied in detail. Future studies should account for differences in nutrient acquisition and resistance to detergents as well as differences in colonization or infection locations (small intestine or colon) when considering whether there are fitness advantages among strains following bile exposure.

SHIGELLA

Shigella species are Gram-negative, facultative, intracellular pathogens that cause a significant global burden each year by causing millions of cases of watery diarrhea or bacillary dysentery, typically in children <5 years of age in developing countries. Infection develops from the ability of the bacteria to invade epithelial cells lining the colon (56, 57). A 220-kb virulence plasmid is required for infection and encodes a T3SS, the Ipa invasion proteins, and additional T3SS effector proteins important for enhancing infection and intracellular survival. As few as 100 cells can cause infection after ingestion. Progression of shigellosis requires the bacteria to transit through the small intestine, where bile exposure occurs, before reaching the site of infection in the colon (56).

Most research involving increased virulence due to bile exposure has focused on *S. flexneri*. The first observations that bile increases the virulence of *S. flexneri* came from an initial study by Pope et al., who identified that adherence, protein secretion, and invasion are induced after the bacteria are exposed to the bile salt deoxycholate (58). Increased adherence was demonstrated to be independent of invasion, since an Δ*ipaB* mutant, which is unable to invade, also had increased adherence to HeLa cells after exposure to bile salts. The virulence factor required for induced adherence was never identified in this study; however, the authors stated that the factor most likely required the T3SS. Increased invasion and adherence were also reported for *Shigella dysenteriae*; however, interestingly, EIEC did not have induced phenotypes following deoxycholate exposure. As stated by the authors, this finding is surprising and warrants further investigation given that the virulence plasmids of *Shigella* and EIEC are highly homologous (58, 59). Indeed, comparisons of the effects of bile and bile salts between *Shigella* and EIEC not only will enhance our understanding of bile-induced virulence for each pathogen but also will allow us to understand key differences between the two pathogens. Further investigation into the effect of bile salts on invasion led to the discovery that deoxycholate interacts with the IpaD invasion protein at the tip of the type III (T3) needle complex. This interaction induces a conformational change to allow another invasion protein, IpaB, to bind, leading to the increased invasion of epithelial cells (60–63). This observation is significant and indicates that IpaD acts as an environmental sensor to prepare the bacteria for induced T3 secretion and subsequent invasion once contact with colonic epithelial cells is made (60). Interestingly, deoxycholate has been utilized to fully investigate the interaction of IpaD and IpaB to elucidate the assembly of the T3 needle complex required for the invasion process (64).

Recently, Faherty et al. identified the *Shigella flexneri* effectors required for bile-induced bacterial adherence (65). By using polarized epithelial cells and a 1:1 mixture of the bile salts cholate and deoxycholate at 0.4% (wt/vol), this study determined that the 99% identical T3SS effector proteins OspE1 and OspE2 were necessary for bile salt-induced adherence, since a double mutant lost the phenotype. The study further demonstrated that *ospE1* and *ospE2* were among the 51 genes induced in response to bile salts, and investigation of the outer membrane identified the localization of the OspE1 and OspE2 proteins following bile salts exposure. Future work will determine the nature of this outer membrane protein localization and to which eukaryotic structures binding occurs. In addition, this study detected increases in T3 and non-T3 protein secretion, as was observed by Pope et al. (58). The mechanism behind the induced protein secretion requires further investigation. It is interesting that in this study, *S. flexneri* was exposed to bile salts during the subculture step, prior to the addition of bacteria to eukaryotic cells in the adherence assay. No differences in adherence were detected when bile salts were added to the cell culture medium during infection (65). This preexposure mimics *in vivo* conditions in that 95% of bile salts are recycled back into the small intestine and do not enter the colon (10). Therefore, these conditions highlight the changes in virulence factor expression that occur during transit in the small intestine prior to invasion at the site of infection in the colon.

In all, studies evaluating the effects of bile salt exposure on *S. flexneri* virulence provide significant evidence that these bacteria respond to environmental changes in the small intestine to enhance virulence prior to reaching the site of infection in the colon. Future work utilizing bile extracts to account for additional factors, analyzing the effects of exposure on other *Shigella* isolates, and determining the mechanisms of bile resistance and bile-regulated virulence will enhance our understanding of the importance of bile in the pathogenesis of *Shigella*.

VIBRIO

Vibrio species are Gram-negative, curved, motile bacteria that are inhabitants of coastal and aquatic environments. Approximately 12 species are pathogenic, with *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* causing the majority of infections, outbreaks, and epidemics worldwide (66, 67). *V. cholerae* is thought to survive in brackish water between epidemics (68), while *V. parahaemolyticus* and *V. vulnificus* are found in raw seafood (69). Infections typically result from the consumption of contaminated food or water and range from mild to severe gastroenteritis characterized by watery diarrhea, severe abdominal cramps, and dehydration (68). Wound infections and septicemia have also been associated with *V. vulnificus* and *V. parahaemolyticus* (70). The bacteria penetrate the mucus layer of the gut and adhere to epithelial cells in the small intestine. Virulence factors required to cause infection include cholera toxin (CT), other enterotoxins, adherence factors such as the toxin-coregulated pilus (TCP), motility systems, and hemolysins (66). The CT and TCP virulence factors are regulated by the transmembrane DNA-binding protein ToxR regulatory cascade (71). ToxR activates the expression of *toxT* in a coordinated effort with TcpP, another transmembrane transcriptional activator. ToxT subsequently activates the transcription of virulence genes, including CT and TCP genes, to facilitate colonization of and disease in the gastrointestinal tract (71, 72). As outlined below, the regulation and coordinated expression of these virulence factors in the presence of bile have enabled *Vibrio* to adapt to and survive within the human host following a lifestyle in aquatic environments.

Vibrio cholerae

V. cholerae represents a well-studied pathogen with regard to bile exposure. There are several mechanisms by which *V. cholerae* resists the bactericidal conditions of bile. First, the general diffusion porins OmpU and OmpT, located in the bacterial outer membrane, have been demonstrated to be critical for bile resistance (72). The expression of *ompU* and *ompT* is mediated by ToxR, which results in induced *ompU* expression but repressed *ompT* expression in the presence of bile salts. It is hypothesized that

OmpU is expressed in the presence of deoxycholate since it is an anion-selective porin that restricts the passage of negatively charged compounds such as those found in bile (73–76). Increased *ompU* expression appears to be unique to *V. cholerae*, as other pathogens such as EHEC and *Salmonella* (see below) repress genes encoding the Omp proteins in the presence of bile. Second, several efflux pumps are required for bile resistance, including the AcrAB-TolC system (77, 78), BreAB (79, 80), VexAB (81), and VprAB (82). Indeed, given the constant exposure of *Vibrio* to bile, the utilization of several efflux pumps is necessary to ensure survival and establish infection in the small intestine. Finally, recent work identified the transcriptional regulator LeuO, also regulated by ToxR, as being important for bile resistance. While the authors this study demonstrated that *leuO* expression was a specific response to bile and required the ToxR periplasmic signaling domain, a clear role for LeuO in bile resistance remains to be identified (75).

Formation of biofilms is a well-characterized phenotype in *Vibrio* species (69). Biofilms are homogenous or heterogeneous communities of bacteria embedded on a matrix of extracellular polymeric substances (EPSs) composed mostly of polysaccharides, with additional molecules such as proteins, lipids, and nucleic acids. Biofilm formation is a common approach for several bacterial pathogens to resist harsh environmental conditions such as exposure to antibiotics, UV radiation, and pH stress (83). Hung et al. investigated biofilm formation in the presence of crude bile and the bile acid sodium cholate. Biofilm formation following sodium cholate treatment was dependent upon the induction of the *Vibrio* polysaccharide synthesis (*vps*) genes and posttranscriptional activation of the regulator VpsR. Moreover, biofilm formation led to an increased ability of bacteria to resist the cytotoxic effects of sodium cholate (84). Recent work demonstrated that the regulation of the second messenger molecule cyclic di-GMP (c-di-GMP) is important for biofilm formation in the presence of bile, as deletion of genes encoding enzymes important for c-di-GMP turnover abolishes biofilm formation (85, 86). Finally, it is worth mentioning that the bile salt taurocholate was recently demonstrated to disperse ingested mature *V. cholerae* biofilms. Since *V. cholerae* biofilms are known to form in aquatic environments, exposure to taurocholate in the proximal small intestine may degrade the environmental biofilm prior to *V. cholerae* virulence gene activation in the new host environment (87). How this observation relates to the above-mentioned observations of biofilm formation in the presence of crude bile and sodium cholate requires further investigation. Nevertheless, *V. cholerae* has clearly utilized biofilm formation to resist the bactericidal conditions of the small intestine and establish infection.

V. cholerae Virulence Factor Expression

The differential expression of several key *V. cholerae* virulence factors following bile exposure has been well documented. One of the more interesting observations is that the *V. cholerae ctxAB* toxin genes and *tcpA*, a component of the TCP, are repressed at high bile concentrations; however, expression is increased once the bacterium reaches the site of infection at the epithelial surface, where the concentration of bile is lower (88, 89). In conjunction with this expression pattern, motility genes are induced significantly in bile and repressed as the bacteria make contact with the epithelium. The authors of this study hypothesized that this coordinated virulence factor expression pattern enables *V. cholerae* to

move to the more permissible colonization environment at the apical surface of epithelial cells, which then triggers CT and TCP expression (88, 89).

The role of ToxR and ToxT in the repression of CT and TCP in the presence of bile is quite complex. Early work demonstrated that ToxT was involved in CT and TCP inhibition (89). However, in light of observations that bile induced OmpU expression through ToxR (72), Hung and Mekalanos performed further analyses to demonstrate that bile acids actually induced *in vitro* CT expression in a ToxR-dependent, ToxT-independent manner (90). These authors stated that the analysis was initiated by the hypothesis that the heterogenous composition of bile had pleiotropic effects on *Vibrio*. The alkaline pH and temperature of 37°C used in this study better represented *in vivo* conditions encountered by these bacteria. Furthermore, a separate study utilizing the *V. cholerae* El Tor biotype, an *ex vivo* intestinal model, and the bile salt taurocholate found that bile salts induced disulfide bond formation in the transcriptional activator TcpP, which in turn led to *toxT* expression and induced virulence (91). While the use of the *ex vivo* model mimics a more physiologically relevant environment for bacteria, the use of the El Tor biotype strain as opposed to the classical biotype strain of *V. cholerae* may explain the different findings regarding ToxT and virulence factor induction. Finally, in light of data from the study by Hung and Mekalanos, further work by Chatterjee et al. with fractionated crude bile demonstrated that the presence of unsaturated fatty acid components of bile, namely, arachidonic acid, linoleic acid, and oleic acid, repressed both the *ctxAB* and *tcpA* genes (92). This repression was mediated through the histone-like nucleoid-structuring transcriptional regulator (H-NS), and recently, it was shown that linoleic acid decreases the binding affinity of ToxT at the promoters of ToxT-controlled genes (93). Motility was also significantly induced in the presence of linoleic acid, which was regulated through the transcriptional activator *flrA* (92). While the individual components of bile have various effects on CT and TCP, bile clearly serves as a signal for *V. cholerae* to optimize virulence factor expression for efficient infection. Indeed, the coordinated virulence factor expression profile in response to bile exemplifies the complex regulatory network required for *V. cholerae* in different environments.

Additional *V. cholerae* virulence factors have been shown to be affected by bile or bile salts. In a TCP/CT-negative clinical isolate of *V. cholerae* belonging to the O39 serogroup, both bile and deoxycholate were found to induce the expression of T3SS genes, including the ToxR-like regulatory genes $vttR_A$ and $vttR_B$ (94). Some of the secreted effectors of the T3SS have been defined, with VopX being shown to be induced by bile and regulated by VttR_A and VttR_B (95). Furthermore, the *trh* gene encoding the related thermostable direct hemolysin (TRH) was induced in the presence of bile, which was regulated by VttR_A, VttR_B, and ToxR (96). A clear role for TRH in *V. cholerae* virulence requires further investigation. Finally, bile acids were recently shown to affect the type VI secretion system (T6SS) of *V. cholerae* (97), which has been demonstrated to be important for environmental competition among bacterial species, particularly in Gram-negative bacteria (98). By using a pandemic strain of *V. cholerae* lacking CT, Bachmann et al. demonstrated that mucin activated the T6SS, but the activity was repressed by deoxycholic acid. Interestingly, cholic acid had no effect on the repression of the T6SS. This study also demonstrated that modification of bile acids by the microbiota

diminished the bacterial killing activity of the T6SS (97). This study not only highlights an important mechanism by which *V. cholerae* competes with commensal bacteria in the gut to establish infection but also demonstrates the importance of intestinal microbiota that modifies bile acids in an attempt to disrupt T6SS-mediated killing. In all, the virulence factors induced during bile exposure clearly demonstrate the ability of *V. cholerae* to alter gene expression in response to host signals to ensure survival and cause disease.

Vibrio parahaemolyticus and *Vibrio vulnificus*

V. parahaemolyticus and *V. vulnificus* are also able to resist the bactericidal conditions of bile. Several efflux pumps required for bile acid resistance have been identified in *V. parahaemolyticus*, including VmeAB, VmeCD, and VmeTUV (99, 100). An analysis of *V. vulnificus* efflux pumps identified VexAB as being important for bile acid resistance despite the lack of significant gene expression induction in the presence of bile acids (101). Additionally, the stress response of the RpoS protein is required for *V. vulnificus* bile resistance (102), and bile-adapted strains have been shown to respond to and survive low-salinity stress (103). These studies highlight mechanisms of bile resistance and tolerance similar to those of *V. cholerae*. As mentioned above, efflux pumps are an important mechanism by which several *Vibrio* species resist bile and adapt to infection in the small intestine, where bile is abundant.

Like *V. cholerae*, virulence factors encoded by *V. parahaemolyticus* include two T3SSs and the accompanying transcriptional regulators, thermostable direct hemolysin (TDH), two flagellar systems, and a T6SS (104, 105). Gotoh et al. demonstrated that the T3SS encoded on chromosome II (T3SS2), which is essential for intestinal colonization and enterotoxicity, was induced during crude bile exposure along with TDH. Furthermore, microarray analysis identified 77 genes induced by bile (105). The induced TDH expression confirms previously reported findings that TDH is secreted more in the presence of bile acids (106). To further highlight the importance of the induced gene profile in *V. parahaemolyticus* during bile exposure, Gotoh et al. demonstrated that absorptive removal of endogenous bile salts in the rabbit ileal loop assay, a measure of enterotoxicity and subsequent intestinal fluid accumulation, significantly reduced fluid accumulation, confirming induced virulence gene expression in the presence of bile. Additionally, these authors determined that the transcriptional regulators VtrA and VtrB were induced by bile and required for induced T3SS2 and TDH expression (105). Recently, an RNA sequencing analysis of *V. parahaemolyticus* exposed to bile and compared to bacteria isolated from an infant rabbit model of infection was performed. Of the 69 genes induced in the presence of bile, 53 were also induced in the rabbit model, and induction of T3SS2 was confirmed (104). These studies not only identify important gene expression profiles of *V. parahaemolyticus* under different conditions but also exemplify how bile exposure can mimic the *in vivo* conditions encountered by bacterial pathogens.

Future Studies of *Vibrio*

Extensive studies of *Vibrio* species related to bile exposure, resistance, and virulence gene activation have allowed a deeper understanding of virulence mechanisms and infections in pathogens that cause a significant global disease burden each year. These studies have also aided researchers who study other bacterial pathogens with similar virulence factors in understanding host-pathogen interactions. Future studies should be directed at further elucidating virulence gene expression of *V. cholerae* following bile exposure, especially given the complex regulatory pathways and variability in the related response to the various components of bile. Indeed, a consensus viewpoint and understanding of virulence gene expression will facilitate future antibiotic and vaccine development. With regard to *V. parahaemolyticus* and *V. vulnificus*, efforts should be focused on improving the comprehension of bile responses and related virulence gene activation. While these pathogens may be similar to *V. cholerae*, identifying differences in the responses to bile for each species may lead to significant advances in the scientific community that will help control infection. Finally, studies that include other *in vivo* signals combined with bile will truly allow researchers to appreciate the complexity of survival in the gastrointestinal tract.

SALMONELLA

Salmonella is a Gram-negative facultative anaerobe with motility conferred by peritrichous flagella. Closely related to *E. coli* but possessing distinct genomic content, there are two *Salmonella* species: *S. bongori* and *S. enterica*. *S. bongori* strains predominantly colonize cold-blooded reptiles, whereas *S. enterica* strains are capable of infecting both humans and mammals (107). *S. enterica* strains fall into six subspecies and are divided into two serovars based on human disease phenotype: nontyphoidal strains cause self-limiting, localized infection through invasion of macrophages and epithelial cells lining the small intestine, while typhoidal strains cause systemic illness due to dissemination to the lymph nodes, liver, and spleen through the lymphatic system (108). Typhoidal strains encompass *S. enterica* serovars Typhi and Paratyphi. Typhoid illness has a clinical presentation consisting of headache, fever, and transient diarrhea or constipation that can lead to systemic pathologies and persistent infection in the respiratory, digestive, or nephrology systems if left untreated. The nontyphoidal *S. enterica* serovars Typhimurium and Enteritidis are represented by numerous subspecies differentiated by O- and H-antigen typing of the lipopolysaccharide (LPS) structure (107, 109). All serovars possess at least five distinct *Salmonella* pathogenicity islands encoding several virulence factors. These genetic elements are denoted *Salmonella* pathogenicity island 1 (SPI-1) to SPI-5, with additional serovar-specific islands, such as SPI-7, encoding the Vi antigen, a capsular polysaccharide that covers the O-antigen of LPS, specific to typhoidal serovars (110–112). The gene clusters encompassed in the pathogenicity islands encode virulence factors such as the immune evasion proteins SptP and SseL; two separate coding regions for T3SSs; and host cell membrane manipulation machinery proteins, including SipA and SopE (113). Despite the fact that typhoidal serovars have smaller genomes than those of nontyphoidal serovars, *S. enterica* serovar Typhi contains additional potent virulence factors such as typhoid toxin and the Vi antigen (112, 114, 115). As highlighted below, bile has been demonstrated to serve as an extremely important physiological stimulus that regulates changes in gene expression for both survival and virulence gene activation.

Salmonella Bile Resistance Mechanisms

The ability of *Salmonella* to survive in bile is a longstanding observation and is utilized in selective media such as MacConkey agar, which contains bile salts, to promote the isolation of *Salmonella* and other Gram-negative bacteria (116, 117). *Salmonella* serovars

TABLE 3 Comparison of *Salmonella* efflux pump genes[e]

Efflux pump gene(s)	Presence in *S. enterica* serovar:			
	Enteritidis[a]	Typhimurium[b]	Typhi[c]	Paratyphi[d]
acrAB	No	Yes	Yes	Yes
acrEF	Partial	Yes	No	Partial
mdtABC	Yes	Yes	Yes	Partial
mdsCBA	Yes	Yes	Yes	Partial
emrAB	Yes	Yes	Partial	Yes
mdtABC	Yes	Yes	Yes	Yes
mdtK	Yes	Yes	Yes	Yes
macAB	Yes	Yes	Yes	Partial
tolC	Yes	Yes	Yes	Yes

[a] Strain P125109 (GenBank accession number NC_011294.1).
[b] Strain LT2 (GenBank accession number NC_003197.1).
[c] Strain CT18 (GenBank accession number NC_003198.1).
[d] Strain ATCC 9150 (GenBank accession number NC_006511.1).
[e] *S. enterica* serovars Enteritidis, Typhi, and Paratyphi carry 78%, 78%, and 56% of the genes carried by *S. enterica* serovar Typhimurium, respectively.

have several approaches for survival in the presence of bile salts, which include defense against membrane destruction and DNA damage facilitated by the detergent activity of bile compounds (3). To prevent bile-mediated destruction, removal of intracellular bile compounds is achieved through the coordinated use of efflux pumps and additional outer membrane proteins. The *S. enterica* serovar Typhimurium genome encodes at least nine efflux pumps for resistance to several antimicrobial compounds, including bile (118). The other *Salmonella* serovars have homologous efflux pump genes; however, there is significant variation between serovars (Table 3). One well-defined efflux pump is the acridine orange removal pump encoded by *acrAB*, an operon that is also carried by EHEC (119, 120). Knockdown of *S. enterica* serovar Typhimurium *acrAB* resulted in hypersensitivity to bile salts, other detergents, and antibiotics (121). Expression of *acrAB* is enhanced after *S. enterica* serovar Typhimurium is exposed to cholic acid, deoxycholic acid, or a mixture of bile salts, a phenotype which was also shown for the EHEC bile response. Regulation of *acrAB* is achieved by activation with RamA or through repression by RamR (119). Interestingly, bile salts stimuli increase the expression of both Ram proteins. Cholate has been shown to directly bind RamA in *Salmonella* Typhimurium, and despite enhanced *ramR* expression under the same conditions, the interaction of RamR with the promoter-binding region is blocked in the presence of bile (119, 122, 123). Additional efflux pump systems identified in *S. enterica* serovar Typhimurium include *acrEF*, *mdtABC*, *mdsCBA*, *emrAB*, *mdtABC*, *mdtK*, and *macAB*, many of which have homologs in *E. coli* or *Pseudomonas aeruginosa* (118). There may be additional efflux pumps that are yet to be identified since no other serovar possesses all nine of the efflux pumps identified in *S. enterica* serovar Typhimurium (Table 2). As seen for *Vibrio*, the constant exposure of *Salmonella* to bile results in the utilization of several efflux pumps to survive and establish infection in the small intestine.

Most efflux pump genes require an associated outer membrane protein in the *tol* family (TolA and TolC) to protect against membrane destruction following bile salts exposure (3). There is significant genetic sequence diversity of the TolA peptide sequences between *S. enterica* serovars Typhi and Typhimurium. *S. enterica* serovar Typhi TolA is 27 amino acids shorter due to the lack of 3

additional tandem repeats in the C-terminal membrane-binding region that exist in *S. enterica* serovar Typhimurium. This amino acid difference suggests a potential explanation for the differences in bile resistance observed between the two serovars. Analysis of the MIC of bile for each serovar demonstrated that the MIC for *S. enterica* serovar Typhimurium is higher (18%) than that for *S. enterica* serovar Typhi (12%) (117, 124). However, dose curve studies indicate that *S. enterica* serovar Typhi may have a survival advantage at higher concentrations of bile (8%) (125). This difference may be due to the ability of *S. enterica* serovar Typhi to survive in biofilms that are frequently observed in the gallbladder of chronic carriers (see below). Nevertheless, deletion of *tolA* in both *S. enterica* serovars Typhi and Typhimurium renders the bacteria susceptible to bile (125). Conversely, NCBI BLAST analysis reveals that the TolC proteins maintain between 99% and 100% amino acid and DNA sequence identities across the sequenced *Salmonella* species, confirming the conserved nature of the structure and function of TolC. Exposure to bile salts stimulates immediate modifications of the outer membrane to prevent additional damage. To enhance the bile-removing processes of the efflux pumps, outer membrane pore-forming complexes are downregulated to prevent additional bile from entering the bacterial cell. For example, reducing the expression of genes encoding the outer membrane proteins OmpF and OmpC, which normally permit bile transport into the bacterial cell, is one strategy by which *Salmonella* prevents bile uptake (3). This strategy is similar to the repression of *ompT* in *Vibrio* in response to bile exposure; however, it remains unknown if *Salmonella* harbors a *V. cholerae* *ompU* homolog for which expression would be increased following bile exposure. Additionally, it has been shown that exposure to the bile salt deoxycholate resulted in a dose-dependent increase in the expression of the regulatory locus *marAB* in *S. enterica* serovar Typhimurium (126), conferring survival in the presence of bile salts and antibiotics. The role of *marAB* is independent of the *acrAB* efflux pump, suggesting that *marAB* regulates a different mechanism for survival during bile-induced stress (4, 126). Taken together, the data establish the importance of efflux pumps and outer membrane pore-forming complexes in maintaining *Salmonella* survival in the presence of bile.

Other modifications to the outer membrane are achieved by targeting LPS. First, the activation of the two-component regulatory system PhoPQ results in the remodeling of the lipid A structure of LPS (3, 117, 127). Although PhoPQ does not sense bile directly, it regulates several genes upon stimulation with bile, including central metabolism and respiration genes (124, 128). Deletion of *phoP* decreases the viability of both *S. enterica* serovars Typhi and Typhimurium in bile, and furthermore, constitutively expressed PhoP increases bacterial fitness in media containing bile (124). However, a recent microarray analysis using physiological murine bile obtained from mice found that *phoP* in *S. enterica* serovar Typhimurium was repressed in the presence of bile following 24 h of exposure, and this repression was independent of PhoPQ signaling. While physiological bovine bile also repressed *phoP* expression, commercial bovine bile and bile acids did not have the same effect. Therefore, different components of bile may be responsible for different responses *in vitro* and *in vivo* (128). Data from this recent analysis parallel the various findings of *Vibrio* research upon the utilization of different sources or components of bile, all of which highlight the importance of the use of physiological conditions to understand the complex interactions

of each pathogen with bile. Certainly, future research analyzing PhoPQ regulation in physiological bile in both *S. enterica* serovars Typhi and Typhimurium will enhance our understanding of this regulation. Second, the addition of aminoarabinose to the lipid A component or the addition of phosphoethanolamine to either the core or lipid A region of LPS alters the outer membrane structure to minimize bile salt destruction (3, 117). Finally, the *fepE* gene of *S. enterica* serovar Typhimurium regulates the production of long O-chains, specifically responsible for the production of O-antigen repeats of >100 units. Mice infected with bacteria lacking the *fepE* gene did not develop colitis as observed with a wild-type strain. The authors of this study hypothesized that modification of the LPS structure through *fepE* may be an important bile survival strategy contributing to *S. enterica* serovar Typhimurium-associated mouse colitis (129). For typhoidal serovars, expression of the Vi antigen capsule is a strategy to mask the LPS structure and prevent immune detection (130). Although expression has not been demonstrated to be regulated by bile exposure, the Vi antigen has been observed in bacteria interacting with the ileal epithelium (131). Future studies should be directed toward determining if bile regulates the expression of the Vi antigen to influence immune evasion prior to contact with host cells. In all, modifications of the bacterial outer membrane occur rapidly after bile exposure to mitigate and prevent damage incurred from bile exposure.

Despite efforts to prevent bile uptake, some bile is still internalized in the bacterial cell, resulting in DNA damage (3, 117). In *Salmonella*, bile exposure increases the frequency of G-C-to-A-T transitions, which has been demonstrated to increase the expression of genes regulated by the stress-induced sigma factors SoxRS and OxyR (132, 133). Specifically, increased expression of DNA adenine methylase (*dam*) after bile salts exposure may serve to repair damage caused by exposure to bile salts, since knockout of *dam* abolished *Salmonella* viability after exposure to bile salts (132). It has also been hypothesized that *S. enterica* serovar Typhi bacteria colonizing the gallbladder in chronic carriers may be more prone to mutagenesis due to the high concentrations of bile salts (133). The constant exposure to high concentrations of bile increases the likelihood of mutagenesis through bile-induced DNA damage. A recent study demonstrated that *S. enterica* serovar Typhi infection of the gallbladder was sufficient to cause gallbladder carcinoma (GBC) in genetically predisposed cells. This association was investigated after the identification of an overlap in populations at risk for both typhoidal infection and GBC, with a high occurrence of both diseases in India (134). Scanu et al. demonstrated that bacterially mediated AKT and mitogen-activated protein kinase (MAPK) activation is required for the initiation of cellular transformation and tumor growth but is not required for sustained tumorigenesis, a conclusion concordant with previously reported hypotheses linking bacterially mediated downregulation of apoptosis and cancer initiation (135). Overall, membrane reorganization to prevent bile salt internalization and DNA damage serves as an important defense mechanism permitting *Salmonella* to survive under bile salt conditions.

Virulence Factor Regulation in *Salmonella*

Exposure of *Salmonella* to bile has been demonstrated to repress the expression of the T3SS through the inhibition of SPI-1, which is essential for bacterial invasion of epithelial cells. The *sirC* and *invF* genes encoding the transcriptional regulators for SPI-1 and SPI-1 effector proteins are downregulated following bile exposure

(3, 117, 136, 137). Since murine infection models reveal *Salmonella* localization to microfold cells (M cells) within Peyer's patches in the ileum, it is believed that areas of high concentrations of bile, such as in the duodenum, do not serve as optimal invasion locations for *Salmonella* (3, 107, 138, 139). Therefore, by repressing the expression of the bacterial invasion machinery at high bile concentrations, *Salmonella* is able to invade cells in a more permissible environment along the gastrointestinal tract, where bile concentrations are decreased (136, 137). Despite data demonstrating the repression of the T3SS in response to bile, the invasion protein SipD, which is the *Salmonella* homolog of the *Shigella* invasion protein IpaD, has been shown to bind bile directly (140, 141). The importance of this binding is not yet known, but it represents a curious observation for how *Salmonella* interacts with the environment during infection. Additional virulence factors repressed by bile in *S. enterica* serovar Typhimurium include the flagellar *flhC*, *flgC*, and *fliC* genes; the fimbrial *fimI* and *fimZ* genes; and other virulence genes (128, 137). Unlike for *Vibrio*, motility appears to be repressed in the presence of bile, which could be linked to gallbladder colonization (see below). Future in-depth analyses linking virulence gene regulation to the duration and composition of bile exposure along the various segments of the gastrointestinal tract will certainly enhance our understanding of *Salmonella* pathogenesis.

Chronic *Salmonella* Infection May Be Attributed to Bile Activation of Survival Genes

Colonization of the gallbladder may serve as a long-term survival strategy for *Salmonella*, as indicated by the downregulation of motility genes and upregulation of stress response genes in response to bile (137). *Salmonella* serovars Typhimurium, Enteritidis, and Typhi have all been isolated from gallbladder biofilms. PCR-based identification of *Salmonella* species in the gallbladder of patients with cholecystitis, a condition characterized by inflammation of the gallbladder, suggests an association between inflammation and gallstone formation (142). Despite the identification of several *Salmonella* serovars in the gallbladder, only *Salmonella* serovar Typhi appears to be capable of long-term survival in the gallbladder, leading to an asymptomatic carrier state that facilitates ongoing bacterial transmission, as exemplified by the famous case of Typhoid Mary (117, 142, 143). Bacteria isolated from gallbladder biofilms show increased expression levels of flagellar filament and fimbrial proteins for colonization of gallstones (117, 137, 142). However, the expression of invasion and motility proteins, such as the flagellar motor, is not necessary for gallstone biofilm formation by *Salmonella* serovars, suggesting that colonization and invasion components are differentially regulated depending on the bile salt concentration of the environment (142). Interestingly, there have been conflicting reports regarding the Vi antigen of *Salmonella* serovar Typhi in gallbladder biofilms. One report demonstrated that EPS matrix production was independent of the Vi antigen, given that a mutant of the regulator for the Vi antigen, a Δ*tviB* mutant, was able to form a biofilm over the course of 18 days (144). However, a recent study demonstrated that active bile-induced biofilms stain positively for Vi antigen expression (145). As stated above, future studies of the regulation of the Vi antigen in the presence of bile will enhance our understanding of this important virulence factor. Certainly, the ability to resist bile and establish a biofilm during gallbladder colonization in the chronic infection state

is essential to the life cycle of *Salmonella* to maintain a survival niche and eventually facilitate transmission to new hosts.

Future Research on *Salmonella*

In summary, while there are several common traits that explain how *Salmonella* serovars respond to and interact with bile, there also appears to be a diverse set of bile responses that distinguishes each serovar. These unique bile responses may serve to provide critical insight into the pathogenicity, infection strategies, and host specificity of each of these diverse serovars. Further research into bile resistance proteins such as TolA, which differ in sequence and function between *Salmonella* serovars Typhimurium and Typhi, will provide insights into bile regulation of *Salmonella* pathogenesis. Conversely, further research into proteins that are homologous between serovars, such as the transcriptional regulator MarA, could reveal critical similarities in gene activation in response to bile. Future research should incorporate *in vivo* models of *Salmonella* colonization and survival under conditions of high and low bile concentrations to enhance our understanding of *Salmonella* infection. Additionally, since bile composition is altered across mammals (146), comparisons of bile compositions among susceptible host species may reveal a host-specific signal responsible for regulating *Salmonella* infection.

CAMPYLOBACTER JEJUNI

Campylobacter jejuni is a Gram-negative, spiral-shaped, microaerophilic bacterium that is a common cause of diarrheal disease around the world. *C. jejuni* colonizes and invades the distal ileum and colonic epithelium, causing cramping, bloody stools, and diarrhea (147). Outbreaks of *C. jejuni* occur in both industrialized and developing nations, typically following the consumption of contaminated food and water; however, developing countries also tend to be areas where the disease is endemic, and high rates of coinfection or asymptomatic carriage are also common (147–149). Although most patients recover after self-limiting infection, 1 in every 2,000 infected individuals develops Guillain-Barré syndrome, an acute disease that leads to muscle weakness and ascending paralysis of the peripheral nervous system and is believed to be an autoimmune response associated with bacterial or viral infections (150). Research with *C. jejuni* has been challenging due to the high degree of genomic plasticity and high rates of genetic transformation found within this naturally competent microorganism (149). However, strains that carry or overexpress flagellum genes, adherence structures, and invasion mechanisms are associated with increased virulence (148–151). As summarized below, pathogenic studies reveal that bile exposure acts as a stimulus for the upregulation of many virulence mechanisms in *C. jejuni*.

Bile Resistance in *C. jejuni*

Similar to other enteric pathogens, *C. jejuni* has developed bile resistance to survive within the human gastrointestinal tract (152, 153). The CmeABC multidrug efflux pump is one of the most studied bile response systems in *C. jejuni* (154). This system is encoded by the *cmeABC* operon, encoding a periplasmic protein (CmeA), an inner membrane transporter (CmeB), and an outer membrane protein (CmeC), respectively. The operon has been demonstrated to be constitutively expressed but is also controlled by a TetR family repressor (CmeR) that recognizes the *cmeABC* promoter region, resulting in decreased transcription (155). Bile salts appear to inhibit the CmeR-*cmeABC* promoter interaction,

which results in increased *cmeABC* operon transcription (153). Once expression is triggered in bile salts, the CmeABC efflux pump increases the ability of the bacteria to withstand the bactericidal conditions of bile, antibiotics, and other antimicrobial substances such as SDS (156). Mutants defective in *cmeABC* expression have a decreased ability to survive in bile salts and colonize chicken intestinal tracts (154). An additional efflux pump, CmeDEF, works in conjunction with CmeABC to confer an additional level of resistance to bile and other antimicrobials (155). However, CmeDEF appears to be a secondary resistance mechanism since bile resistance is dominated by the activity of CmeABC, and CmeDEF may preserve bile resistance only in the absence of CmeABC (157).

Additional mechanisms implicated in the *C. jejuni* bile response are two-component regulatory systems (TCRSs) that sense and respond to bile salts. TCRS are often utilized by bacteria after exposure to environmental stimuli as a means of altering gene expression to cope with changing conditions (158). Raphael et al. identified the *Campylobacter* bile resistance regulator (CbrR) through *in silico* analyses and subsequent genetic mutagenesis experiments (159). Response regulators, such as CbrR, are vital TCRS components that either directly or indirectly alter gene expression after phosphorylation via a histidine/sensor kinase receptor (158). A CbrR mutant could not survive in the presence of medium supplemented with bile salts and had a diminished ability to colonize chickens (159). Although the CbrR-binding partners and the exact mechanism of bile resistance are unknown, studies have also demonstrated that the response regulator has a critical role in *C. jejuni* survival in bile. Recently, Okoli et al. utilized a bioinformatic approach with protein and domain architecture analyses to assess genomes of members of the order *Campylobacterales* for the presence of homologous proteins important for bile responses in other bacteria (152). This study identified 151 proteins conserved across species, as well as species-specific genes, implicated in bile tolerance. This cross-species analysis also revealed putative proteins that may be important for the *C. jejuni* bile response (152). Future studies will determine if these genes encode proteins that regulate virulence mechanisms in *C. jejuni* in the presence of bile.

C. jejuni Virulence Factor Expression in the Presence of Bile

In addition to survival and resistance genes, bile exposure increases the expression of key virulence factors in *C. jejuni*. The *Campylobacter* invasive antigens (Cia) are proteins secreted directly into the epithelial cell cytoplasm through a flagellar apparatus, resulting in host membrane ruffling, alterations in signaling and intracellular trafficking, and increased bacterial uptake (160–162). Mutagenesis studies that blocked Cia secretion revealed significantly reduced *C. jejuni* internalization by epithelial cells (161). The Cia proteins are thought to be synthesized and secreted in response to stimulatory substances provided by the host. Moreover, the Cia proteins have been induced after exposure to bile acids such as deoxycholate, cholate, and chenodeoxycholate (163, 164). Exposure to other molecules, such as heat-stable proteins in fetal bovine serum, epithelial cell lysates, and extracellular matrix components, has also been found to increase the synthesis of Cia. However, secretion of the Cia proteins through the flagellar apparatus appears to be dependent on bacterial contact with cellular components of host epithelia (164).

Two additional virulence mechanisms in *C. jejuni* that may be regulated by bile exposure are biofilm formation and motility

genes. One study identified that flagella are necessary for enhanced *C. jejuni* biofilm formation following bile salts exposure, with the flagellar filament mediating attachment for the biofilm (165). Another study used proteomic two-dimensional (2D) gel electrophoresis to demonstrate that ox bile may increase the expression of flagellin A, the chemotaxis protein CheV, and the ATP synthase, suggesting that bile components may induce motility and act as chemotactic attractants for *C. jejuni* (166). Li et al. further confirmed that bile components are chemotactic attractants after observing that methyl-accepting chemotaxis proteins are needed for chemotaxis and that the absence of these proteins, or the absence of bile, severely impairs colonization in mice (167). In contrast, a study examining motility via zone-of-migration assays found that deoxycholate exposure did not alter the motility of *C. jejuni in vitro*, and deoxycholate did not affect adherence to epithelial cells (168). These divergent findings for both bile salt-dependent motility and adherence could be due to the genomic plasticity and phase variation found in *C. jejuni* isolates. Future research is needed to elucidate the relationships that bile exposure has to the colonization, internalization, and motility of *C. jejuni*. Furthermore, transcriptomic assessment of bile salts exposure over various time points is needed to describe the impact of bile on *C. jejuni* pathogenesis during biofilm formation and planktonic stages.

GRAM-POSITIVE BACTERIA

While the majority of enteric pathogens studied in detail are Gram-negative bacteria, a few Gram-positive bacteria can cause a significant burden of infection in the gastrointestinal tract. This section highlights recent findings of studies investigating bile responses in *Clostridium difficile* and *Listeria monocytogenes*. As outlined below, and like Gram-negative pathogens, these Gram-positive bacteria are able to survive and adapt in the presence of bile while also enhancing infection and survival in the gastrointestinal environment.

Clostridium difficile

C. difficile is a spore-forming bacterium responsible for recurring gastrointestinal colitis associated with recent antibiotic treatment (169). Several *C. difficile* studies have demonstrated that bile has a significant impact on *C. difficile* germination, which can be either enhanced or inhibited by different bile acids. Primary bile acids have been shown to promote the germination of *C. difficile* spores, while secondary bile acids inhibit the germination of spores into vegetative bacteria (170–172). Although only 5% of the bile acids along the gastrointestinal tract typically enter the colon in a healthy individual, during *C. difficile* infection, bile composition and microbiota homeostasis are significantly altered to increase the levels of primary bile acids in the colon and potentially promote the germination of *C. difficile* spores (29, 173). Antibiotic treatment perturbs the commensal microbiota that converts the primary bile acids into secondary bile acids, which normally inhibit *C. difficile* proliferation (170, 172, 173). The loss of the microbiota therefore results in a greater abundance of primary bile acids that can germinate *C. difficile* spores (170, 172). However, the increased concentration of primary bile acids in the colon during recurring *C. difficile* infections can be reversed following fecal microbiota transplantation (173). Much like other enteric bacteria, *C. difficile* appears to have found a mechanism for overcoming the inhibitory effects of bile by exploiting the dynamics between the compositions of microbiota and bile in the gastroin-

TABLE 4 Future bile-related research goals for each pathogen

Pathogen	Future research goal(s)
Escherichia coli	Analysis of additional pathovars such as EIEC, AIEC, and DAEC
	Further analysis of bile resistance and regulation of bile-mediated virulence
	Further analysis of commensal organisms
ETEC	Functional studies to characterize the bile response and coordination of virulence factor expression
EPEC	Characterization of the mechanism and clinical implications of bile malabsorption during infection
EHEC	Future transcriptomic studies to understand the complete bile response
Shigella	Identification of resistance mechanisms and regulation of virulence factors
	Utilization of bile extract in future analyses
Vibrio	
V. cholerae	Further investigation into the complex regulatory network of the bile response
	Use of consistent sources of bile
V. parahaemolyticus	Improved understanding of the bile response
	Further analysis of bile-mediated virulence
V. vulnificus	Identification of virulence factors affected by bile exposure
Salmonella	Comparison of serovars to understand various bile responses and related infection strategies
	Utilization of bile in analyses of infection models
Campylobacter	Clarification of the relationship between bile exposure and induction of adherence and motility
	Additional transcriptomic analyses of bile exposure
Clostridium	Identification of resistance mechanisms and additional virulence factors
Listeria	Identification of virulence factors affected by bile exposure

testinal tract. It will be interesting to see if additional *C. difficile* virulence factors are induced by bile.

Listeria monocytogenes

L. monocytogenes is a foodborne pathogen that causes large outbreaks due to colonization of prepared or prepackaged food sources such as deli meat and produce and exists in the environment as a saprophyte that can gain access to food sources either directly or through carriage in farm animals (174). This pathogen can efficiently adapt to the different host environments, including the gastrointestinal tract (174, 175). As reviewed by Gahan and Hill, bile induces stress responses (176), efflux pumps and related transcriptional regulators (177), as well as a bile salt hydrolase enzyme (178), with each factor contributing to *L. monocytogenes* survival during bile exposure (174). The bile stress response includes the induced expression of DNA repair enzymes, chaperones, and oxidative stress proteins (176, 179). Furthermore, a recent study compared the abilities of different isolates to survive bile exposure under aerobic and anaerobic conditions at acidic

pH. White et al. demonstrated that acidic pH reduced bile resistance but that anaerobic conditions improved bile resistance at pH 5.5, particularly in virulent isolates from humans. The authors hypothesized that oxygen availability may influence stress response genes (180), which requires further investigation. Finally, numerous studies have identified gallbladder infection and colonization in both animal models and, in rare cases, human gallbladder infections. It is hypothesized that growth in the bile-rich environment of the gallbladder enhances fecal shedding during infection (174). Taken together, the survival of and potentially enhanced infection by *L. monocytogenes* as a result of bile exposure highlight the ability of this pathogen to maintain a successful infectious life cycle. Future work identifying additional virulence factors induced by bile will enhance our understanding of the virulence mechanisms of this foodborne opportunistic pathogen.

CONCLUDING REMARKS

In closing, enteric pathogens, and even some commensal bacteria, have adapted to the environment of the gastrointestinal tract to efficiently colonize the host and cause infection. As a whole, enteric bacteria have modified outer membrane proteins, utilized porins, and relied on efflux mechanisms to resist the bactericidal conditions of bile. It is fascinating how unrelated species have utilized similar mechanisms, particularly with regard to efflux pumps, as a prime example of convergent evolution. Furthermore, as these bacteria are able to survive in the presence of bile, enteric pathogens have evolved to exploit this host signal to regulate virulence factor gene expression. Whether each species activates virulence genes to colonize the small intestine or delays the expression of virulence factors until a more permissive environment in the ileum, colon, or epithelial surface of the gastrointestinal tract is reached, the coordination of virulence gene expression in the presence of bile is quite remarkable and provides fertile ground for further studies using the global transcriptional analysis tools of RNA-Seq. The studies outlined here have highlighted the ability of these pathogens to adapt to and successfully colonize the host and cause infection. As researchers move forward with investigative studies (Table 4) and vaccine development for enteric pathogens, we hope that future experiments will utilize conditions that better represent the *in vivo* environment encountered by these bacteria. Even the simple addition of bile or bile salts to culture media has significantly enhanced our understanding of enteric bacterial pathogenesis. We hope that the gains in the awareness of bile-regulated virulence in enteric bacteria will finally result in successful vaccine development for many of these formidable pathogens.

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